

Contribution of Malate and Amino Acid Metabolism to Cytoplasmic pH Regulation in Hypoxic Maize Root Tips Studied Using Nuclear Magnetic Resonance Spectroscopy¹

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ABSTRACT

³¹P-, ¹³C-, and ¹⁵N-nuclear magnetic resonance spectroscopy were used to determine the roles of malate, succinate, Ala, Asp, Glu, Gln, and γ -aminobutyrate (GABA) in the energy metabolism and regulation of cytoplasmic pH in hypoxic maize (*Zea mays* L.) root tips. Nitrogen status was manipulated by perfusing root tips with ammonium sulfate prior to hypoxia; this pretreatment led to enhanced synthesis of Ala early in hypoxia, and of GABA at later times. We show that: (a) the ability to regulate cytoplasmic pH during hypoxia is not significantly affected by enhanced Ala synthesis. (b) Independent of nitrogen status, decarboxylation of Glu to GABA is greatest after several hours of hypoxia, as metabolism collapses. (c) Early in hypoxia, cytoplasmic malate is in part decarboxylated to pyruvate (leading to Ala, lactate, and ethanol), and in part converted to succinate. It appears that activation of malic enzyme serves to limit cytoplasmic acidosis early in hypoxia. (d) Ala synthesis in hypoxic root tips under these conditions is due to transfer of nitrogen ultimately derived from Asp and Gln, present in oxygenated tissue. We describe the relative contributions of glycolysis and malate decarboxylation in providing Ala carbons. (e) Succinate accumulation during hypoxia can be attributed to metabolism of Asp and malate; this flux to succinate is energetically negligible. There is no detectable net flux from Glc to succinate during hypoxia. The significance of the above metabolic reactions relative to ethanol and lactate production, and to flooding tolerance, is discussed. The regulation of the patterns of metabolism during hypoxia is considered with respect to cytoplasmic pH and redox state.

production during the first several hours of hypoxia. Fermentation of glucose to ethanol may or may not lead to cytoplasmic acidosis, depending on whether or not the coproduct carbon dioxide can escape the tissue (18). In the absence of a build-up of cytoplasmic carbon dioxide during prolonged hypoxia, cytoplasmic acidification due to leakage of protons from vacuoles is apparent (18).

Although the aforementioned metabolic reactions may explain significant features of metabolism in certain hypoxic plants, a number of questions remain concerning other metabolites. First, the decarboxylation of glutamate to GABA² has been suggested to play a role in limiting cytoplasmic acidosis in maize root tips (13). Second, the mechanism and function of malate consumption in hypoxic roots is unclear (6, 25, 26). Third, fermentation of glucose to Ala has long been recognized as a significant process (6, 26, 28)—comparable in magnitude to fermentation to lactate—yet the contribution of Ala production to cytoplasmic acidosis is ambiguous. Fourth, production of succinate has been proposed to be involved in limiting cytoplasmic acidosis (11, 12). Here we address each of the above questions, by describing the metabolism of Gln, Glu, Ala, Asp, GABA, succinate, and malate in hypoxic maize root tips, using NMR spectroscopy (16). The integrated regulation of this metabolism, and the production of lactate and ethanol, is discussed with respect to redox state and cytoplasmic pH.

MATERIALS AND METHODS

Plant Material

Maize (*Zea mays* L. Funk hybrid 4323 from Germain's Seeds, Los Angeles, CA) was soaked for 1 d in flowing deionized water, then covered with wet paper towels in a tray that was covered with aluminum foil. After approximately 48 h, root tips (2 mm long) were excised on ice with a razor blade. Each sample contained approximately 2 g of root tips. Root tips were rinsed in deionized water to remove root cap slime, and transferred to a 10 mm diameter NMR tube, modified to permit perfusion (15).

Perfusion Conditions

In all experiments, root tip samples were initially perfused with 100 mL of oxygenated medium that was recirculated for

Cytoplasmic acidosis in seedling root tips has been shown to be an important determinant of tolerance of severe hypoxia (18, 19). For maize, cytoplasmic acidosis early in hypoxia has been correlated with fermentation of glucose to lactate (17). This acidification triggers a switch to ethanolic fermentation—the principal fermentation product in maize root tips that contain sufficient alcohol dehydrogenase activity (17, 20). Maize root tips deficient in alcohol dehydrogenase synthesize lactate throughout hypoxia, exhibit severe cytoplasmic acidosis, and are much less tolerant of hypoxia than wild type (18, 20). Similarly, Menegus *et al.* (11) found that plant species that are more tolerant of hypoxia exhibit limited lactate

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² Abbreviation: GABA, γ -aminobutyric acid.

3 h at 10 mL/min (pretreatment). The pretreatment perfusion medium contained 50 mM Glc (^{31}P - and ^{15}N -NMR experiments) or $[1\text{-}^{13}\text{C}]\text{Glc}$ (^{13}C -NMR experiments) in 0.1 mM CaSO_4 , 10 mM Mes (brought to pH 6.5 with Tris), plus the antibiotics gentamycin and amphotericin B at 50 and 2.5 mg/L, respectively. Ammonium pretreatment consisted of supplementing the oxygenated medium with various concentrations of ammonium sulfate (^{15}N -enriched for the ^{15}N -NMR experiments). During these oxygenated pretreatments, the labeling of intracellular metabolites with ^{13}C from $[1\text{-}^{13}\text{C}]\text{Glc}$ was in near steady-state, for the intensities of NMR signals from these metabolites was constant after approximately 2 h of pretreatment.

Hypoxic treatment consisted of perfusion with nitrogen-saturated 0.1 mM CaSO_4 at 4 mL/min, without recirculation; no glucose or ammonium was included. Glucose was not included in the hypoxic perfusion medium because it has little effect on metabolic activity in Glc-pretreated root tips during the first several hours of hypoxia, as reflected in ethanol production (19). ^{13}C - and ^{15}N -enriched chemicals (at >99 atom %) were obtained from Isotech, Miamisburg, OH). Experiments were carried out at room temperature.

NMR Spectroscopy

All spectra were obtained using a General Electric GN500 spectrometer. ^{31}P - and ^{13}C -NMR spectra were obtained at 202.5 and 125.7 MHz, respectively, exactly as described previously (2), under nonsaturating conditions. ^{15}N -NMR spectra were obtained at 50.6 MHz with bilevel proton decoupling using the WALTZ pulse sequence. The interval between pulses was approximately 3 s, spectral width 6000 Hz, 8 k data points. Twenty-Hz line broadening was applied to spectra prior to Fourier transformation. Resonances were assigned based on correspondence with chemical shifts of standards. The relative abundance of ^{13}C in metabolites observable by ^{13}C -NMR was quantitated as follows (*cf.* 16). First, spectral peak areas were measured by manually cutting and weighing each peak. Some metabolite peaks overlapped with "natural abundance" signals from intracellular polymers, in which case they were corrected accordingly. Peak areas were also corrected for the nuclear Overhauser effect, determined for each resonance in cell extracts. These corrections allowed us to quantitate the relative amounts of ^{13}C in the different carbons of each metabolite. As noted above, the perfusion conditions led to near-steady-state in ^{13}C -labeling of metabolites, so that changes in the relative abundance of ^{13}C in the various metabolites reflect proportionally the changes in total pool sizes of each metabolite.

Metabolite Extraction, Fractionation, and Assay

At the end of NMR experiments, samples were frozen in liquid nitrogen. Low mol wt metabolites were extracted with 5% HClO_4 , followed by centrifugation and neutralization with KOH, prior to analysis by NMR. Organic acids and amino acids were isolated by ion-exchange chromatography using Bio-Rad AG1-X8 (formate) and AG50W-X8 (hydrogen ion), respectively. Amino acids were eluted from the cation exchanger with 4 N ammonium hydroxide; malate was eluted

from the anion exchanger with 2.5 N formic acid. Ethanol in the perfusion effluent, and lactate and alanine in tissue extracts, were assayed enzymatically (1).

RESULTS

Ammonium Pretreatment Enhances Production of Ala and GABA in Hypoxic Maize Root Tips

The objective of these experiments was to alter the capacity of root tips to make Ala and GABA, and thereby determine what impact production of these metabolites can have on energy metabolism and cytoplasmic pH. If Ala production involved production of protons, tissues exhibiting elevated Ala production during hypoxia might be expected to also exhibit greater cytoplasmic acidosis. And if Glu decarboxylation to GABA is important in preventing cytoplasmic acidosis, tissues exhibiting enhanced production of GABA might be expected to maintain higher cytoplasmic pH during hypoxia.

We used ^{13}C -NMR spectroscopy to monitor the production of Ala and GABA in root tips labeled with $[1\text{-}^{13}\text{C}]\text{Glc}$ (*cf.* 17). Pretreatment of root tips with 2.5 mM ammonium sulfate (5 mM ammonium ion) increased Ala synthesis more than two-fold during subsequent hypoxia (Fig. 1). This enhanced Ala synthesis was associated with a slight (approximately 25%) reduction in the amount of lactate synthesized during hypoxia (Figs. 1 and 2). Ammonium pretreatment also increased levels of GABA during hypoxia, relative to controls, in which GABA was not detected after 90 min of hypoxia (Fig. 2, peaks 5, 6, and 12; here GABA was analyzed in extracts because the resolution and sensitivity of signals is greater than for *in vivo* NMR). However, GABA accumulation was observed in control root tips after several hours of hypoxia (data not shown). We found no significant effect of ammonium pretreatment on either the maximum flux to ethanol, or the lag-time between the onset of hypoxia and the onset of ethanol production (*cf.* 17) (data not shown).

Enhanced Production of Ala and GABA in Maize Root Tips Has Negligible Impact on the Ability to Regulate Cytoplasmic pH during the First 90 min of Hypoxia

We measured cytoplasmic pH in maize root tips using ^{31}P -NMR (16, 24). Pretreatment of root tips with 5 mM ammonium ion has little effect on cytoplasmic pH changes after the onset of hypoxia (Fig. 3). In similar experiments using different ammonium sulfate pretreatment concentrations, only at 20 mM exogenous ammonium (10 mM ammonium sulfate) was significantly greater cytoplasmic acidosis observed during subsequent hypoxia (data not shown). This result may be due to toxic effects of the ammonium ion, including its ability to collapse transmembrane pH gradients (7, 10).

An initial overshoot in cytoplasmic acidosis, in which cytoplasmic pH at 15 min of hypoxia is lower than at 40 min, is evident in Figure 3. Such an overshoot has been observed in some of our earlier work (18). However, in other studies we saw a rapid initial acidification, followed by a period of relative stability in cytoplasmic pH (*e.g.* 17). We attribute observation of an overshoot to greater metabolic synchrony within the tissue samples, possibly due to a sharper transition from oxygenated to nitrogen-saturated perfusion medium. A

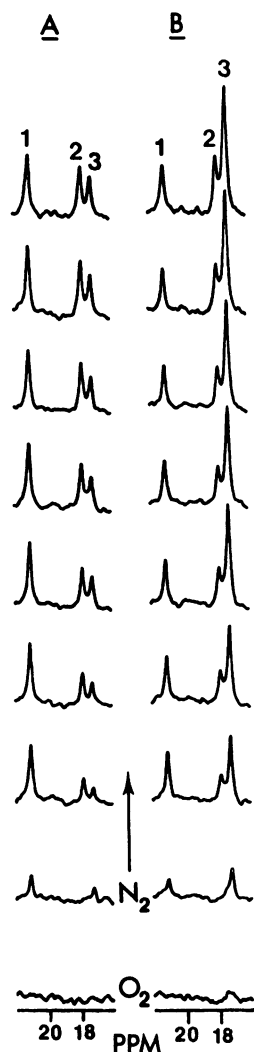


Figure 1. Enhanced Ala synthesis during hypoxia in ammonium-pretreated maize root tips. *In vivo* ^{13}C -NMR partial spectra of methyl resonances in root tips pretreated with (B) or without (A) 2.5 mM $(\text{NH}_4)_2\text{SO}_4$, prior to the onset of hypoxia (see "Materials and Methods"). Spectra were obtained every 10 min, sequentially from bottom to top. Resonances are assigned to lactate (peak 1), ethanol (peak 2), and Ala (peak 3). The amounts of lactate and Ala in nonammonium-pretreated root tips after 40 min of hypoxia, determined enzymatically in extracts, are, respectively, approximately 3 and 2 $\mu\text{mol/g}$ tissue (data not shown).

more gradual transition would deplete oxygen in root tips at different rates, thus "smearing-out" metabolic responses over the sample and obscuring any transient behavior.

The results in Figures 1 through 3 indicate that cytoplasmic pH in maize root tips during the first 90 min of hypoxia is not altered significantly in tissues in which synthesis of Ala and GABA are greatly enhanced. Either the reactions that generate these amino acids do not produce or consume cytosolic protons, or any such protons are masked by other reactions involving protons. This issue is addressed in the "Discussion." The remainder of the "Results" section de-

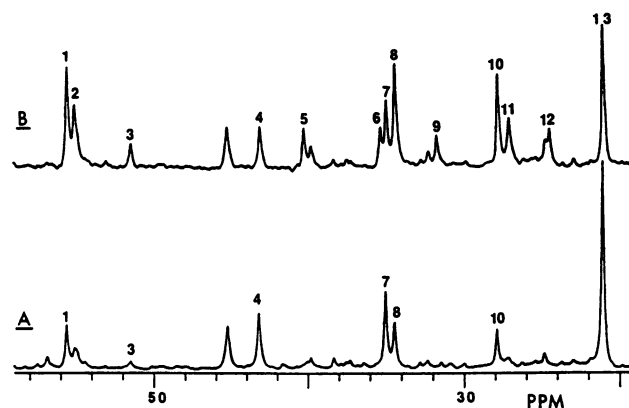


Figure 2. Enhanced GABA synthesis during hypoxia in ammonium-pretreated maize root tips. ^{13}C -NMR partial spectra of extracts of root tips prepared after 90 min of hypoxia. Root tips were pretreated either with (B) or without (A) 2.5 mM $(\text{NH}_4)_2\text{SO}_4$. Peak assignments: 1, C2-Glu; 2, C2-Gln; 3, C2-Ala; 4, C3-malate; 5, C4-GABA; 6, C2-GABA; 7, C2/C3-succinate; 8, C4-Glu; 9, C4-Gln; 10, C3-Glu; 11, C3-Gln; 12, C3-GABA; 13, C3-lactate.

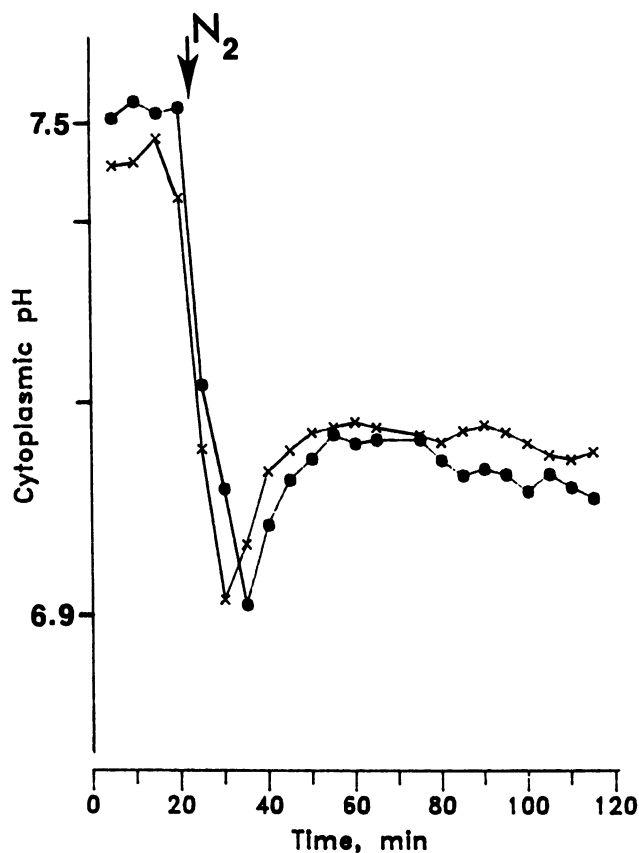


Figure 3. Effect of ammonium pretreatment on cytoplasmic pH in maize root tips during hypoxia, determined *in vivo* by ^{31}P -NMR. Root tips were pretreated with (●) or without (×) 2.5 mM $(\text{NH}_4)_2\text{SO}_4$ for 3 h, then switched to nitrogen-saturated perfusion medium, as indicated by the arrow. Cytoplasmic pH was estimated from the chemical shift of the cytoplasmic Pi resonance, in consecutively acquired 5 min spectra, as described previously (16, 17).

scribes changes in the patterns of carbon and nitrogen metabolism during hypoxia.

In Ammonium Pretreated Maize Root Tips during Hypoxia, Carbons in Gln Are Initially Converted to Glu, and Nitrogen from Gln and Asp Is Metabolized to Ala; Later, Conversion of Glu to GABA Predominates

We followed the fate of carbons in metabolites derived from $[1-^{13}\text{C}]\text{Glc}$, using *in vivo* ^{13}C -NMR. Oxygenated root tips exposed to ammonium contain a high level of Gln, which declines during the first hour of hypoxia as Glu increases (Figs. 4 and 5). Later, Glu declines along with Gln, such that after 6 h of hypoxia, levels of Glu have returned to values found prior to the hypoxic treatment, and the net decline in Gln is matched by the increase in GABA (Fig. 5). It would thus appear that the carbons of Gln, Glu, and GABA act as a discrete metabolic unit during hypoxia.

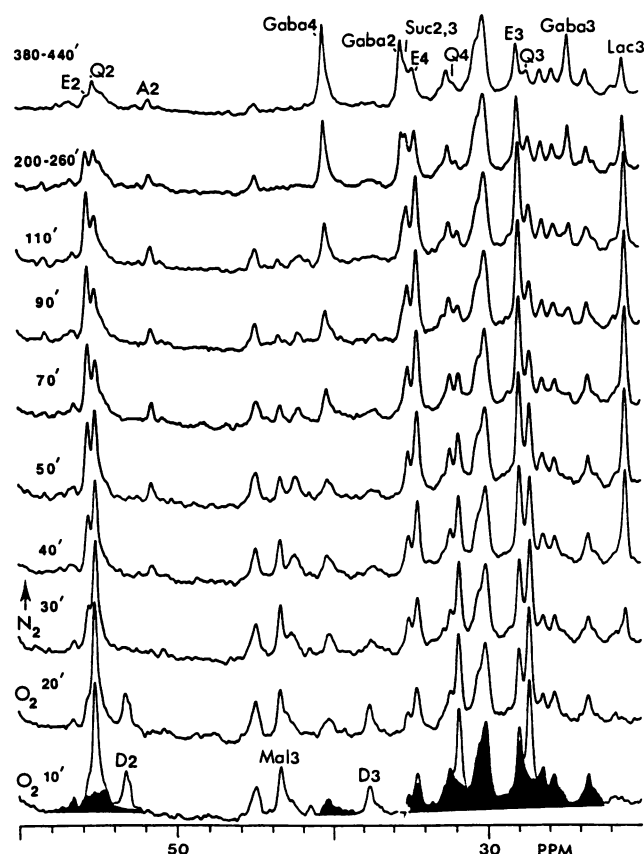


Figure 4. *In vivo* ^{13}C -NMR partial spectra of maize root tips perfused for 3 h with oxygenated medium containing 2.5 mM $(\text{NH}_4)_2\text{SO}_4$ (bottom two spectra), followed by hypoxia (remaining spectra). Spectra were accumulated bottom to top, at the times indicated; the bottom five spectra were the sum of individual 10 min spectra from six replicate experiments; the next three spectra were summed from individual 20 min spectra from three replicate experiments; the remaining 1 h spectra were from a single experiment. Solid (black) peaks are due to natural abundance ^{13}C resonances (from cellular polymers). Peak assignments: E, Glu; Q, Gln; A, Ala; D, Asp.

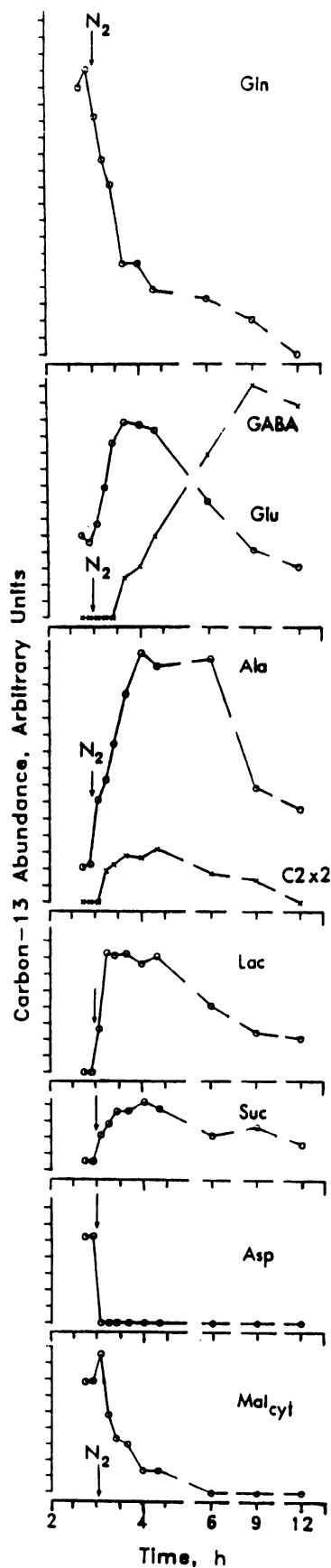
The conversion of carbons in Gln to Glu and GABA requires net disposal of the amide nitrogen on Gln. This nitrogen is transferred ultimately to Ala; the increase in Ala during the first hour of hypoxia can be accounted for by the consumption of Gln and Asp (Fig. 5). ^{15}N -NMR experiments confirmed the net transfer of Gln-amide groups to Ala, and also demonstrated that net conversion of intracellular ammonium into Ala was insignificant under the experimental conditions described here (data not shown).

Although ammonium pretreatment enhances synthesis of both Ala and GABA during subsequent hypoxia, the time courses for accumulation of these metabolites are quite distinct. Most Ala is synthesized during the first hour of hypoxia (Figs. 1 and 5), whereas GABA was not detected until 40 min of hypoxia (Figs. 4 and 5). GABA reaches its highest level after several hours of hypoxia, at a time when levels of all other metabolites are declining (Figs. 4 and 5) because intracellular sugar pools run out (data not shown). This behavior is quite similar to that reported for radish leaves (28).

In nonammonium-pretreated root tips, Gln is less than one-third as abundant as Glu, hence the reduced synthesis of Ala during hypoxia in this tissue (Fig. 1). The low level of Gln in nonpretreated root tips also accounts for the lower levels of Glu and GABA early in hypoxia (compare Fig. 2A and B). Levels of other metabolites, and the metabolic changes described in the next section, were quite similar in ammonium- and nonammonium-pretreated root tips. The most significant differences were approximately 25% lower levels of Asp and Glu, and approximately 25% higher levels of malate, in nonammonium-pretreated root tips prior to the onset of hypoxia (data not shown).

Early in Hypoxia, Carbons of Asp and Malate Are Metabolized in Part to Succinate and in Part to Ala, Lactate, and Ethanol, via Pyruvate

Aspartate disappears virtually immediately with the onset of hypoxia (Figs. 4 and 5). The carbons of Asp appear to be funneled into the Krebs cycle, because both malate and succinate increase during the first 10 min of hypoxia. After 10 min of hypoxia, cytoplasmic malate declines, disappearing after approximately 90 min. This decrease in cytoplasmic malate is accompanied by a further increase in succinate, and by the appearance of $[2-^{13}\text{C}]\text{Ala}$. Because glycolysis of $[1-^{13}\text{C}]\text{Glc}$ produces $[3-^{13}\text{C}]\text{pyruvate}$ and, with transamination, $[3-^{13}\text{C}]\text{Ala}$ (8, 17), these reactions cannot account for the presence of $[2-^{13}\text{C}]\text{Ala}$. We attribute $[2-^{13}\text{C}]\text{Ala}$ synthesis to conversion of $[2-^{13}\text{C}]\text{malate}$ to $[2-^{13}\text{C}]\text{pyruvate}$ by malic enzyme, followed by transamination, for the following reasons. First, maize root tips fed $[1-^{13}\text{C}]\text{Glc}$ contain significant levels of $[2-^{13}\text{C}]\text{malate}$. *In vivo* $[2-^{13}\text{C}]\text{malate}$ signals are obscured by carbohydrate signals. However, analysis of extracts shows that malate is labeled at either C2 or C3 to very similar extents (with much lower abundance at C1 and C4) (data not shown; cf. 3). Thus, the intensity of the $[2-^{13}\text{C}]\text{malate}$ resonance *in vivo* can be inferred from the intensity of the $[3-^{13}\text{C}]\text{malate}$ resonance. In principle, the action of malate dehydrogenase, phosphoenolpyruvate carboxykinase, pyruvate kinase, and transaminase could also produce $[2-^{13}\text{C}]\text{Ala}$ from $[2-^{13}\text{C}]\text{malate}$. However, maize root tips contain little phosphoenolpy-



ruvate carboxykinase (4), so such a sequence is unlikely to be significant *in vivo*. Second, accumulation of $[2-^{13}\text{C}]\text{Ala}$ is coincident with depletion of malate (Fig. 5). Third, activity of malic enzyme should increase after cytoplasmic acidosis, because this enzyme has a low pH optimum (5).

Conversion of $[2-^{13}\text{C}]\text{malate}$ to $[2-^{13}\text{C}]\text{pyruvate}$ early in hypoxia is also indicated by the presence of $[2-^{13}\text{C}]\text{lactate}$ (chemical shift 69.4 ppm) observed *in vivo* and in cell extracts and $[1-^{13}\text{C}]\text{ethanol}$ (chemical shift 58.4 ppm) observed *in vivo*. In spectra accumulated over the first 60 min of hypoxia, these lactate and ethanol resonances were, respectively, approximately 4 and 3% the intensity of the ^{13}C -methyl resonances from these molecules (data not shown).

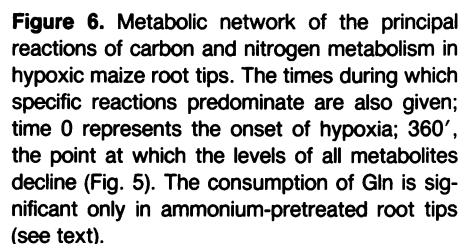
DISCUSSION

Paths of Carbon and Nitrogen in Hypoxic Maize Root Tips and Metabolism of Cytoplasmic Protons

The demonstrated hypoxia-induced decreases in Asp, Gln, and malate, and the increases in ethanol, lactate, Ala, Glu, GABA, and succinate, can be accounted for by the scheme in Figure 6. The reactions presented are balanced for charge, and so for protons. Adenine nucleotide and phosphate interconversions were ignored, because changes in the pool sizes of these metabolites (21) are small relative to the changes in organic and amino acid pool sizes examined here. Fermentation of Glc to ethanol leads to no net production of protons. Fermentation of Glc to lactate, or Glc/Gln to Ala/Glu, results in net production of two protons per mol Glc. Likewise, synthesis of Ala and succinate from Asp and Glc results in production of two protons per mol Glc; however, if flavin adenine dinucleotide produced with succinate were regenerated by cytoplasmic NADH/H^+ via ubiquinone and the glycerol phosphate shuttle (29), the net proton balance would be zero. In contrast, reactions leading to the net stoichiometric consumption of protons in the physiological pH range are: (a) conversion of malate to lactate or ethanol, or malate/Gln to Ala/Glu; (b) GABA synthesis from Glu; and (c) conversion of carbons and nitrogen of Asp to Ala.

The differences between metabolism in ammonium-pretreated and nonpretreated root tips can be largely attributed to the more than 10-fold higher levels of Gln at the end of ammonium pretreatment. The small pool of Gln in root tips not fed ammonium precludes any large increase in Ala, Glu, or GABA during the first 90 min of hypoxia (Figs. 1 and 2).

Figure 5. Hypoxia-induced changes in the relative abundance of ^{13}C in the predominant three, four, and five carbon acids in maize root tips. Abundances were derived from peak areas of NMR signals from each metabolite (see "Materials and Methods"). The onset of hypoxia is indicated by arrows. The two time courses for Ala represent twice the abundance of $[2-^{13}\text{C}]\text{Ala}$ (\times) (i.e. the amount of Ala synthesized from malate or Asp, because malate/Asp are equally labeled at C2 and C3) and the abundance of $[3-^{13}\text{C}]\text{Ala}$ due solely to glycolysis and transamination (\circ) (i.e. the abundance of $[3-^{13}\text{C}]\text{Ala}$ minus the abundance of $[2-^{13}\text{C}]\text{Ala}$, because $[2-^{13}\text{C}]\text{Ala}$ synthesis is concomitant with synthesis of an equal amount of $[3-^{13}\text{C}]\text{Ala}$ from or via malate). The ^{13}C abundance in malate was determined as twice the abundance of $[3-^{13}\text{C}]\text{malate}$ (see text).



The integrated flow of carbon and nitrogen in the reactions shown in Figure 6 can be viewed as a response in which redox state and cytoplasmic pH dominate regulation, as follows. First, the intracellular redox state becomes more reduced within 2 min of the onset of hypoxia, as oxidative phosphorylation ceases (17). The greater reducing potential of pyridine nucleotide redox couples will favor increases in [lactate]/[pyruvate], [Glu]/[Gln], [succinate]/[fumarate], and [malate]/[oxaloacetate]. (Pyridine nucleotide redox pairs are postulated to be coupled to mitochondrial flavin nucleotide redox states, via reactions such as the glycerol-3-phosphate shuttle and the ubiquinone pool.) Second, glutamine synthetase is inhibited by elevated AMP and ADP, and by lower ATP and ammonium (27). The above can account for accumulation of lactate, succinate, malate, conversion of Gln to Glu, and conversion of Asp to C4 acids (principally succinate and malate) via transaminase. Of the enzymes responsible for these interconversions, transaminase and malate dehydrogenase are clearly the most active, given the rapidity of Asp depletion (Fig. 5). Third, and finally, is the activation of pyruvate decarboxylase (17) and malic enzyme (5) by low pH. Activation of pyruvate decarboxylase after approximately 10 min of hypoxia ensures continued ATP synthesis via fermentation to ethanol (the principal fermentation end product in hypoxic maize root tips), without concomitant cytoplasmic acidosis (17). The activation of malic enzyme after approximately 10 min of hypoxia leads to net depletion of cyto-

The large cytoplasmic acidification that takes place during the first 15 min of hypoxia (Fig. 3) is coincident with three different acid-producing fermentations: Glc to lactate; Glc/Gln to Ala/Glu; and Glc/Asp to Ala/malate/succinate (Fig. 6, and discussion above). Nonammonium-pretreated root tips accumulate approximately 3 μmol lactate and approximately 1 μmol Ala/g tissue over this period (Figs. 1 and 5), yielding approximately 4 μeq of cytoplasmic protons. The net hydrolysis of approximately 0.3 μmol MgNTP to NDP, which occurs within the first 2 min of depletion of tissue oxygen (21), would increase this total acid production by no more than 10%, considering the H^+ stoichiometry for ATP hydrolysis at alkaline pH (9). The intracellular buffering capacity of maize root tips is approximately 14 μeq H^+ /g tissue (23), of which approximately half is cytoplasmic; the remainder can be assigned to the vacuole, based on ^{31}P -NMR measurements of vacuolar Pi content in maize root tips (22). Therefore, we conclude that for nonammonium-pretreated root tips, the production of lactate and Ala can account for the initial 0.5 to 0.6 pH unit drop in cytoplasmic pH (Fig. 3).

10–75 min

After approximately 15 min of hypoxia, accumulation of lactate and depletion of Asp is complete (Fig. 5), and in nonammonium-pretreated root tips the pool size of Gln is insufficient to contribute to further Ala synthesis. In ammonium-pretreated root tips, cytoplasmic acid production continues to total approximately 6 μmol cytoplasmic H^+ by 75 min, due to fermentation of Glc/Gln to Ala/Glu and Glc to lactate (Figs. 1, 5, and 6). Measurements of vacuolar pH, based on the chemical shift of $[3\text{-}^{13}\text{C}]\text{malate}$ (*cf.* 2, in which cytoplasmic and vacuolar ^{13}C -NMR signals from malate carboxylic acid groups are resolved), indicate that some of these cytoplasmic protons are transported into vacuoles. This may in part explain why the ability to regulate cytoplasmic pH early in hypoxia is not dependent on nitrogen status (Fig. 3). These measurements of vacuolar pH, the results of which will be reported elsewhere, also revealed effects of ammonium pretreatment on vacuolar pH.

A second reaction responsible for removing cytoplasmic protons takes place after approximately 10 min of hypoxia, regardless of nitrogen status, *viz.* the decarboxylation of malate. Decarboxylation is revealed by the presence of C2-labeled Ala and lactate, and C1-labeled ethanol, and is attributed to activation of malic enzyme by cytoplasmic acidosis (5). Oxygenated maize root tips contain approximately 2.2 μmol malate/g tissue (3). Aspartate can also provide substrate for malic enzyme during hypoxia, via transaminase and malate dehydrogenase (Fig. 6). The level of Asp in oxygenated root tips is approximately 70% that of cytoplasmic malate (Fig. 5), for a total of approximately 3.7 μmol malate/Asp per g tissue. Approximately 1 $\mu\text{mol/g}$ of this is converted to Ala (Fig. 5); approximately 0.25 $\mu\text{mol/g}$ to lactate (determined from the relative intensities of $[2\text{-}^{13}\text{C}]$ - and $[3\text{-}^{13}\text{C}]\text{lactate}$ described in "Results," and lactate contents given in Fig. 1); approximately 1.5 $\mu\text{mol/g}$ to ethanol (determined from the relative intensities of $[1\text{-}^{13}\text{C}]$ - and $[2\text{-}^{13}\text{C}]\text{ethanol}$ described in "Results," and a flux to ethanol during the first hour of hypoxia of approximately 25 $\mu\text{mol/g}$ (17); and approximately 1 $\mu\text{mol/g}$ to succinate (Fig. 5). The time of greatest malate depletion and $[2\text{-}^{13}\text{C}]\text{Ala}$ synthesis (Fig. 5) coincides with the small cytoplasmic pH increase observed between approximately 15 and 35 min of hypoxia (Fig. 3). We conclude that sufficient malate/Asp is decarboxylated (approximately 2.75 $\mu\text{mol/g}$) to account for this increase. Thus, we propose a significant role for malate in the regulation of cytoplasmic pH in hypoxic plant tissues; malate decarboxylation, coupled with the activation of pyruvate decarboxylase and initiation of ethanol production (17, 18), permits rapid cessation of the cytoplasmic acidosis caused by fermentation of Glc to lactate and Ala. The importance of this function is also suggested by the decrease in malate observed in a wide variety of plant species and tissues early in hypoxia (6, 25, 26).

30–360 min

Much later in hypoxia comes a further acid-consuming reaction, decarboxylation of Glu to GABA. GABA is first seen in significant amounts in ammonium-pretreated root tips after 30 min of hypoxia (Fig. 5). Production of GABA in

the first hour of hypoxia may partially counteract cytoplasmic proton accumulation due to enhanced Ala synthesis, together with proton transport from cytoplasm to vacuole and decarboxylation of malate. However, GABA synthesis is greatest after the predominant acid-producing reactions have ceased. In nonammonium-pretreated root tips, GABA synthesis is negligible in the first 90 min of hypoxia (Fig. 2), and so cannot contribute to cytoplasmic pH regulation, contrary to the suggestion of Reid *et al.* (13). After several hours of hypoxia, net Glu catabolism is evident, in both ammonium-pretreated and non-pretreated root tips. Decarboxylation of Glu proceeds such that GABA becomes the predominant intracellular metabolite. These results suggest that GABA accumulation, in excess of other metabolites, could be used as a metabolic marker for dead or dying hypoxic plant cells. Consistent with this view are the results of Menegus *et al.* (11), who showed that, after 8 h of anoxia, the highest levels of GABA are found in plant species that are least tolerant of low oxygen.

Absence of Fermentation to Succinate during Hypoxia

Recent investigations of anaerobic metabolism in various cereals (11, 12) and algae (30) have drawn attention to succinate as a metabolic end product. Our results here show that succinate accumulation in maize root tips during hypoxia is simply a consequence of Asp and malate metabolism, and is not due to a fermentation reaction. Hence, succinate is not a significant factor with respect to either cytoplasmic pH regulation or energy production. These points argue against a role for succinate in resistance to hypoxia. Consistent with this view are the data of Menegus *et al.* (11), who compared metabolite levels in shoots of several species under air and after 8 h of anoxia, and found remarkably similar levels of succinate despite a wide range of sensitivities to low oxygen among the different species tested. At later times in hypoxia, after tissues of other crop plants have died, rice and the rice-weed *Echinochloa* continue to accumulate more succinate (11); the mechanism and significance of this accumulation are unclear.

Although the role of succinate in resistance of plants to low oxygen appears unsubstantiated, there is no shortage of alternative traits that might confer resistance. One is the decarboxylation of arginine observed in rice shoots, but not roots, during anaerobiosis (14). On the one hand, such reactions might influence cytoplasmic pH, as does malate decarboxylation in maize root tips. On the other, arginine decarboxylation might be simply a response (not an antidote) to cytoplasmic acidosis, as glutamate decarboxylation in maize root tips appears to be from the present study. A second candidate is the limited production of lactate in rice shoots during anoxia (11), which suggests that pyruvate decarboxylase is activated at higher cytoplasmic pH levels in rice, so that less cytoplasmic acidification is required to switch on ethanol production, compared with cereals such as maize and wheat. Such comparisons of different species and tissues, and under different "anaerobic" conditions, undoubtedly have the potential to become searches for oversimplification. Nevertheless, the further analysis of metabolism in these different situations, with respect to regulation of cytoplasmic pH, may continue to provide a useful perspective.

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